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Alteration of cytosolic calcium induced by angiotensin II and norepinephrine in mesangial cells from diabetic rats

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Alteration of cytosolic calcium induced by angiotensin II and norepinephrine in mesangial cells from diabetic rats. To evaluate functional alterations of mesangial cells induced by diabetes (DMC), we observed the changes of cytosolic calcium ([Ca]i) in response to the vasoconstrictor agonists angiotensin II (Ang II) and norepinephrine (NOR). DMC were obtained from rats with streptozotocin-induced diabetes, cultured in normal medium and identified as mesangial cells (MC) in the third subculture. [Ca]i was measured using fura-2 as a fluorophore. Basal calcium levels (60 to 80 nM) in DMC were not different from control mesangial cells (CMC). The high glucose (30 mM) medium concentration reduced the response of CMC and DMC to Ang II and NOR. This was not an osmotic effect since mannitol did not alter these responses. When DMC were stimulated with Ang II, a desensitized response was always observed, with a transient variation of [Ca]i ($N = 6, P < 0.05$). In contrast, a non-desensitized response with a sustained pattern of [Ca]i increases was obtained in NOR-stimulated DMC. Therefore, the present results suggest that DMC show a modified response to stimulation of the Ang II receptor, which is expressed phenotypically in culture by desensitization. Furthermore, these alterations induced by diabetes environment in MC *in vivo* were maintained *in vitro* despite a long period (~5 months) in which the cells were grown in normal culture medium.

Diabetic nephropathy is a late complication occurring in approximately 35 to 45% of patients with insulin-dependent diabetes mellitus (IDDM) [1], with the glomerular mesangium being the most affected region [2].

Glomerular hyperfiltration is the first hemodynamic manifestation of the disease [3]. The decreased sensitivity of afferent arterioles to vasoconstrictors, together with a decrease in the contractile ability of mesangial cells and the increased production of vasodilating substances with maintained efferent arteriole reactivity [4], explain the finding of hyperfiltration with glomerular hypertension that later progresses to a reduction in glomerular filtration by an extrinsic compression of the glomerular capillary loops secondary to an expansion of the mesangial matrix, and culminates with end-stage chronic renal failure [4].

Countless factors have been studied in an attempt to elucidate the causal factor of glomerular hyperfiltration. Greater emphasis has been placed on hemodynamic causes and more recently on the metabolic changes induced by chronic hyperglycemia. Angiotensin II (Ang II) has always attracted considerable interest because it is known that the use of angiotensin converting enzyme inhibitor reverses glomerular hyperfiltration and improves the course of the disease [5]. Kikkawa et al [6] demonstrated that the glomeruli of diabetic rats have a lower contractile ability when stimulated with Ang II, a fact possibly explaining the phenomena of glomerular hyperfiltration. In a study of mesangial cells from normal rats cultured in the presence of high glucose levels, Menè et al [7] demonstrated a lower contractile response to Ang II. On the other hand, glomeruli stimulated with noradrenaline (NOR) presented a normal contractile capacity [4], although studies on vascular smooth muscle have led to conflicting results ranging from decreased to exacerbated reactivity [8].

Ang II and NOR receptors belong to a superfamily of membrane receptors linked to guanosine triphosphate (GTP) or guanine nucleotide-binding proteins (G protein) [9]. The activation of these receptors induces activation of G protein, which for Ang II exists in two forms: a monomeric form, still little studied [10], and a heterotrimeric form consisting of the α, β and γ subunits [10]. Subunit α consists of two families: G αi, which inhibits adenylate cyclase, and G αq, which activates C phospholipase (PLC) [11]. Although the descriptions of G proteins for NOR are still unclear, they are known to activate PLC leading to the formation of inositol triphosphate (IP3). Therefore, Ang II and NOR utilize the same secondary messenger pathway (IP3) for the transduction of the membrane signal.

IP3 is a secondary messenger primarily acting by promoting the release of calcium ions sequestered inside the sarcoplasmatic reticulum (SR) [12], which together with diacylglycerol activate cytosolic enzymes such as protein kinase C (PKC) and stimulate calcium influx through the membrane. Besides this effect, there is an activation of the negative feedback of cytosolic calcium ([Ca]i) and PKC elevation that inhibits the hydrolysis of membrane phospholipids, thus reducing the free IP3 pool [13–15] and [Ca]i.

A dysfunction of calcium metabolism has been reported to occur in untreated insulin-dependent diabetes mellitus (IDDM) [16]. Platelets from non-controlled diabetic patients in a hyperglycemic state present higher basal calcium levels than control cells due to inhibition of membrane Na/K-ATPase [17]. In contrast, cells of vascular smooth muscle and mesangial cells in culture exposed to high glucose concentrations have been shown to present normal basal calcium levels. However, when stimulated with vasoconstrictors such as Ang II, they present a lower elevation in cytosolic calcium [7]. These findings are reversed by

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PKC blockers, demonstrating that PKC is activated by a mechanism of receptor down-regulation, perhaps due to a higher glucose concentration in the extracellular medium [7].

Since no studies exist in the literature about the behavior of mesangial cells in culture from diabetic rats when stimulated with vasoactive hormones, the objective of the present investigation was to specifically evaluate the changes in cytosolic calcium induced by Ang II and NOR in mesangial cells from untreated rats with 60-day induced diabetes in the presence of normal or high glucose levels.

Methods

IDDM induction

Young adult Wistar male rats weighing 230 to 350 g were divided into two groups: control and streptozotocin induced diabetes (IDDM). The animals of both groups were placed in cages with free access to food and water and observed for 60, 120 and 180 days.

IDDM was induced with a single dose of streptozotocin (STZ), 50 mg/kg body wt, diluted in 0.3 ml 0.01 M trisodium citrate buffer and administered intravenously through the caudal vein. During the subsequent 48 hours, 5% aqueous glucose solution was provided to all rats and blood glucose levels were read with a glucometer I apparatus. Animals whose readings were 400 mg/dl (24.5 mM) or more were considered to be diabetic and were left in their cages with free access to food and water for 60 days without insulin replacement. Control animals were maintained under the same conditions for 30, 60, 120 and 180 days. On the 30th, 60th, 120th, and 180th days the control and IDDM animals were weighed and their tail arterial pressure (TAP) was measured.

Twenty-four hour microalbuminuria was determined by radial immunodiffusion [18] and blood glucose levels were measured. The animals in the diabetic group whose glucose levels were 400 mg/dl (24.5 mM) or more were submitted to nephrectomy for isolation of the glomeruli. Since no significant differences were observed in the results obtained on the different days of observation, only the data for the 60-day group are shown to avoid repetition. The data obtained for both groups of rats are presented in Figure 1, which shows loss of weight with increased mean tail pressure and microalbuminuria in STZ induced rats.

Mesangial cell culture

Normal and diabetic rats ($N = 6$ per group) considered to be adequate for the experiment were anesthetized with ethyl ether and submitted to bilateral nephrectomy. The kidneys were decapsulated and cortical macrodissection was performed. The cortex was fragmented and forced through three sieves in decreasing order of mesh size (60, 100 and 200 mesh). The glomeruli were then collected from the surface of the third sieve and forced through a 25 × 7 gauge needle for full decapsulation. The material was collected into a conic tube with a cap and centrifuged at 475 g/two minutes. The supernatant was discarded and the precipitate resuspended in a collagenase solution, 750 U/4 ml unsupplemented culture medium (RPMI 1640), and incubated at 37°C in an atmosphere humidified with 5% CO_2 for 30 minutes, with shaking at five minute intervals. After incubation, the material was centrifuged at 25 g for two minutes, the supernatant was discarded and the precipitate resuspended in RPMI 1640 supplemented with 20% fetal calf serum. The cultures were allowed to develop in a CO_2 incubator until they reached 100% confluence. The medium was replaced with fresh medium supplemented with 20% serum, 2 mM glutamine and 0.3 g crystalline penicillin G at 48 hour intervals. At the time of 100% confluence, the cultures were submitted to trypsinization with 0.2% trypsin-EDTA and subcultured in flasks under the same culture conditions. This procedure was repeated up to the third subculture, when the cells were

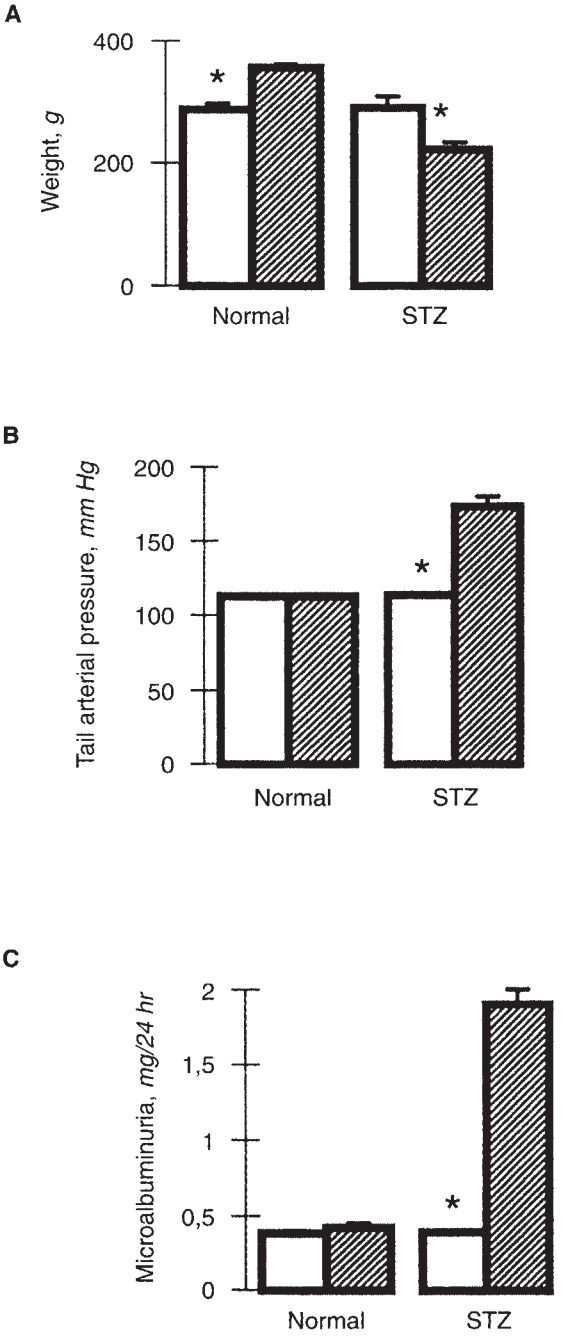

Fig. 1. *Body weight* (**A**), *tail arterial pressure* (**B**) *and 24 hour microalbuminuria* (**C**) *in normal* ($N = 6$) *and STZ-diabetes* ($N = 6$) *groups.* Symbols are: (□) baseline at the beginning of the experiments; (▨) at the end of 60 days. All values are means ± SE of 6 experiments; $^*P < 0.05$. Some error bars are so small that they are included in the bars.

prepared for the experiment. Although not shown, different subcultures from the first to the tenth were tested with Ang II and NOR and the same responses to these agonists were observed up to eight subcultures. The third one was chosen to avoid cellular contaminants and later subcultive phenotypic alterations. For the high-glucose group, 48 hours after the third trypsinization the cells were exposed to culture medium containing 30 mM glucose and the medium was changed at 24 hour intervals for 72 hours, when the cultures were trypsinized for cytosolic calcium measurement and left to stand for 24 hours without serum.

Mesangial cell characterization

Mesangial cells were characterized by phase microscopy, showing a polygonal shape (stellate cells). Electron microscopy showed numerous intracellular microfilaments in their prolongations. The cells were also characterized by their immunofluorescent staining with several primary antibodies: (a) positive immunofluorescence staining for actin and myosin monospecific antiserum; (b) immunofluorescence staining of the extracellular matrix for type IV collagen and fibronectin using monospecific antiserum; (c) negative for antibodies to human factor VII antigens and keratin, a fact that excluded the possibility endothelial and epithelial cells, respectively.

Cytosolic calcium

Calcium was measured after cell incubation with the fluorescence indicator fura 2 in the form of acetoxymethylester (AM). Mesangial cells cultured in the third passage at a concentration of 10^6 cells/ml were resuspended in 2.5 ml Tyrode (137 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$ $2H_2O$, 0.49 mM $MgCl_2$ $6H_2O$, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, and 5.5 mM D-glucose) containing 0.2% bovine serum albumin and left to stand in a CO_2 incubator at 37°C for 30 minutes. The suspension was then centrifuged at 100 g for four minutes, and the supernatant was aspirated and the pellet resuspended in 2.5 ml albumin-free Tyrode and transferred to the quartz cuvette of a SPEX fluorimeter (AR CM System, NJ, USA) for autofluorescence determination. Measurements were made at 340 and 380 nm excitation wavelengths, with emission at 505 nm. The autofluorescence ratio was less than 10% and therefore was not subtracted from the fluorescence measurements before calculation of the fluorescence ratio. The cells were then incubated with 0.01% Pluronic 127 detergent and 2 μM fura-2/AM and the cuvette was transferred to a Perkin Elmer spectrofluorimeter (LS 5B, Buckinghamshire, UK) to record the fluorescence spectrum of the indicator in the excitation range of 300 to 400 nm, with emission at 520 nm. In the esterified form, maximum fluorescence was observed at 390 nm. As the indicator was transformed to the acid form fura-2, the fluorescence peak shifted to the 350 nm wavelength within a mean period of three hours, thus indicating the maximum amount of indicator incorporation into the cell suspension. At that time the cell suspension was washed with 15 ml Tyrode and centrifuged at 100 g for four minutes. The supernatant was discarded and the pellet resuspended in 2.5 ml Tyrode and transferred to a SPEX fluorimeter programmed for excitation at two wavelengths (340 and 380 nm) with emission at 505 nm, under constant stirring at 37°C. The first reading of this phase corresponded to basal calcium. The cells were then stimulated with increasing doses of Ang II (10^{-7}, 2×10^{-7}, 5×10^{-7}, 10^{-6}, 5×10^{-6} and 10^{-5} M) or NOR (10^{-9}, 5×10^{-9}, 10^{-8}, 5×10^{-8}, 10^{-7}, 5×10^{-7}, 10^{-6}, and 5×10^{-6} M). At the end of each experiment, a control was performed using 50 μM digitonin, 1 mM manganese and 2 mM EGTA. The results are reported as the relative ratio of the 340 and 380 nm wavelengths, considering the reading for digitonin to be 100%, and calcium concentration was estimated by the formula of Grynkiewicz, Poenie and Tsien [19].

The following six groups of cells from normal and diabetic animals were incubated under different osmotic conditions before (72 hr) to the calcium studies: normal mesangial cells cultured in 11 mM glucose, 30 mM glucose or 30 mM mannitol and stimulated with increasing doses of Ang II or NOR, and diabetic mesangial cells stimulated as done for their control group (diabetic animals). Six replications were performed for each condition.

Chemicals

Fura-2/AM and Pluronic were purchased from Molecular Probes (Eugene, OR, USA), and glucose and mannitol from Merck (Darmstadt, Germany). All other products were supplied by Sigma Chemical Co. (St. Louis, MO, USA).

Statistical analysis

Data were analyzed statistically by parametric and nonparametric tests depending on the variable or on the variability of the measurements made. The *t*-test was used for the *in vivo* parameters, and the Wilcoxon, Mann-Whitney or Kruskal-Wallis tests were used for the *in vitro* tests. Values less than 0.05 were considered to be significant, and they are presented as mean ± SE.

Results

At the end of the 60-day observation period, the control rats presented a body wt gain (from 287 ± 10 to 375 ± 5 g, $P < 0.05$) and were normotensive (TAP of 112 ± 1 and 112 ± 1 mm Hg, NS) and their microalbuminuria levels were within normal limits (0.38 ± 0.02 and 0.42 ± 0.03 mg/24 hr, NS). In contrast, diabetic rats presented a body wt loss (from 290 ± 20 to 222 ± 11 g, $P < 0.05$) and were hypertensive (from 113 ± 1 to 173 ± 7 mm Hg, $P < 0.05$), with microalbuminuria from 0.39 ± 0.01 to 1.90 ± 0.1 mg/24 hr ($P < 0.05$; Fig. 1 A-C), from day 0 to day 60.

Basal calcium

The mean estimated basal calcium concentration in the presence of 1.36 mM calcium in the extracellular medium for the mesangial cells of control rats cultured in the presence of 11 mM glucose did not differ from the value obtained from cells cultured in the presence of 30 mM glucose (Fig. 2). The same was observed for diabetic cells. When the basal calcium values of normal and diabetic cells cultured in the presence of 11 or 30 mM glucose were compared, no significant difference was observed.

Angiotensin II

As shown in Figure 3A, the mesangial cells from normal rats presented increased fluorescence intensity with a progressive increase of Ang II dose, and therefore the effect obtained with the lowest dose was lower than the effect obtained with the maximum dose ($P < 0.05$) for cells cultured in the presence of both 11 and 30 mM glucose. In contrast, a desensitization of the response was observed in diabetic mesangial cells since the maximum effect was obtained with the lowest dose administered ($P < 0.05$). The successively administered progressive doses of Ang II did not increase the fluorescence intensity (Fig. 4). A transient variation

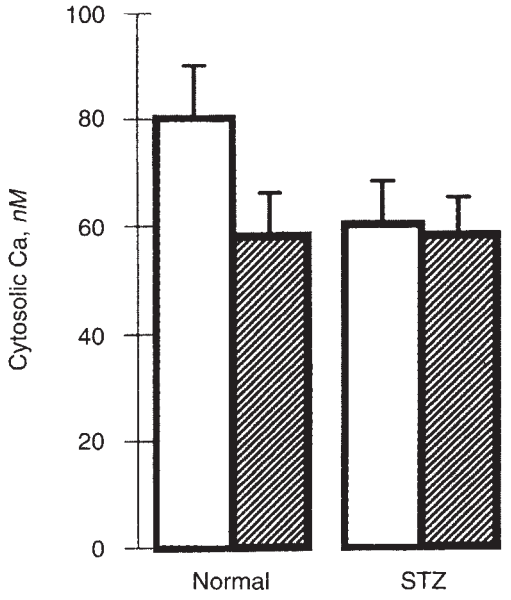

Fig. 2. *Effect of high glucose (Glc) on basal cytosolic calcium of cultured mesangial cells.* Symbols are: (□) physiological culture medium (11 mM glucose, $N = 18$) and (▨) cells cultured in the presence of 30 mM glucose ($N = 21$). All the values are the means of estimated basal calcium concentration ± SE, calculated from fluorescence measurements. No significant difference was observed.

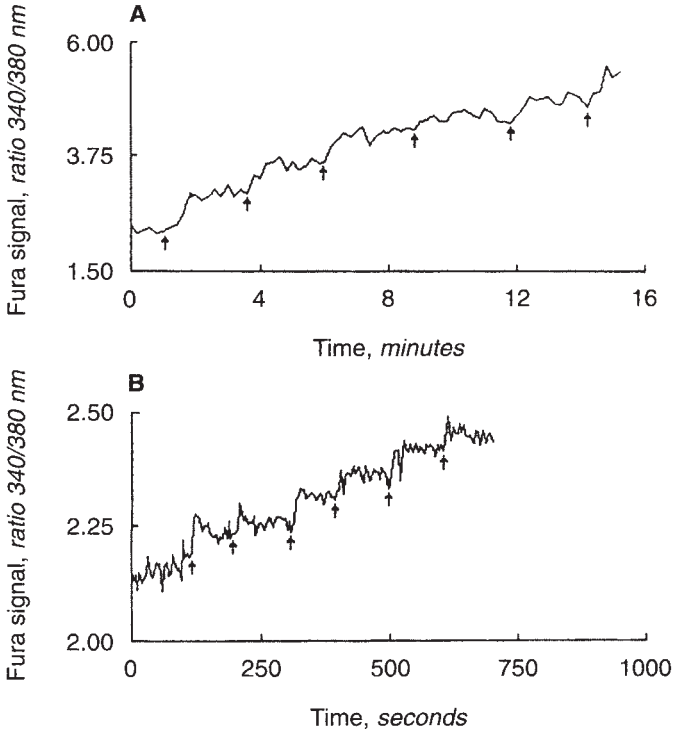

Fig. 3. *Typical experiment comparing the effects of Ang II on cultured mesangial cells from normal Wistar rats grown in* (**A**) *physiological (11 mM) and* (**B**) *high (30 mM) glucose (Glc).* Traces reflect changes in the fura signal (340/380 nm ratio) induced by increasing doses of Ang II (10^{-7}, 2×10^{-7}, 5×10^{-7}, 10^{-6}, 5×10^{-6} and 10^{-5} M) indicated by the arrows.

was compared to the sustained increase observed in the control rat cells for both glucose concentrations (Fig. 3 A, B).

When comparing the cytosolic calcium concentration obtained with the maximum and minimum Ang II doses, the mesangial cells of control rats responded with greater intensity than the diabetic cells. Also, both control and diabetic cells responded with greater intensity when cultured in the presence of 11 mM glucose than when cultured in the presence of 30 mM glucose ($P < 0.05$, Test molecular wt; Fig. 5).

Control and diabetic mesangial cells cultured in the presence of mannitol did not modify the pattern of response to Ang II (results not shown).

Norepinephrine

Mesangial cells from control rats cultured in the presence of 11 or 30 mM glucose responded with a sustained increase in cytosolic calcium when stimulated with progressive NOR doses (Fig. 6). The same pattern of variation in response was also observed in diabetic cells (Fig. 7). However, when control and diabetic cells were compared in terms of the intensity of the variation in response, diabetic cells were found to present a lower variation in the increase of the fluorescence intensity in relation to control cells ($P < 0.05$). Moreover, when the cells cultured in the presence of 11 and 30 mM glucose were compared in terms of the variation in levels of cytosolic calcium, the cells cultured in the presence of 11 mM glucose were found to present a greater variation in the elevation of cytosolic calcium compared to cells cultured in the presence of 30 mM glucose ($P < 0.05$; Fig. 8).

Mannitol did not modify the pattern of response to NOR in control or diabetic cells (results not shown).

Discussion

Calcium ion is an important mediator of cell signaling phenomena and is essential for cell contraction. In mesangial cells, intracellular calcium ion is regulated by a fine mechanism of calcium influx, efflux and Ca uptake/release by SR. The calcium influx depends on voltage-dependent calcium channels, on non-specific cationic channels or on calcium release-activated channels (CRAC) [20]. Efflux is regulated by the membrane Ca-ATPase together with the Na/Ca exchanger, which act to guarantee the calcium efflux into the extracellular medium. The Ca sequestered into the SR is mobilizable through receptor activation of Gq and consequent production of IP3, which is the common pathway for the response to Ang II and NOR. Whenever an increase in calcium ion concentration occurs, the contractile proteins of the mesangial cytoskeleton are activated, permitting mesangial cell contraction with modification of glomerular hemodynamics through the alteration of the glomerular ultrafiltration coefficient (K_f) [21].

Under resting conditions, the basal calcium of diabetic cells did not differ significantly from that of normal cells either in the presence of normal or elevated glucose ($N = 21$, $P > 0.05$). Similarly, Menè et al [7], working with mesangial cells from normal rats in the presence of high glucose levels, did not observe changes in basal calcium levels; however, when the mesangial cells were stimulated with Ang II they presented a lower response in elevating cytosolic calcium.

Under Ang II stimulation, the mesangial cells from diabetic rats (Fig. 4) presented a sharply different behavior compared to Ang

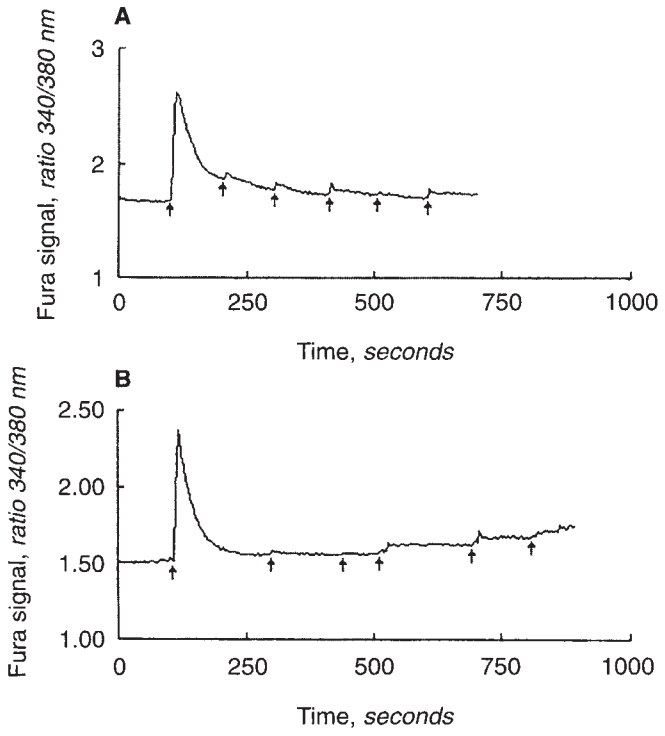

Fig. 4. *Typical experiment comparing the effects of Ang II on cultured mesangial cells from STZ-diabetic rats grown in* (**A**) *physiological (11 mM) and* (**B**) *high (30 mM) glucose (Glc).* These traces are representative of 6 experiments each, of cumulative dose-response curves. Arrows indicate addition of Ang II (10^{-7}, 2×10^{-7}, 5×10^{-7}, 10^{-6}, 5×10^{-6} and 10^{-5} M).

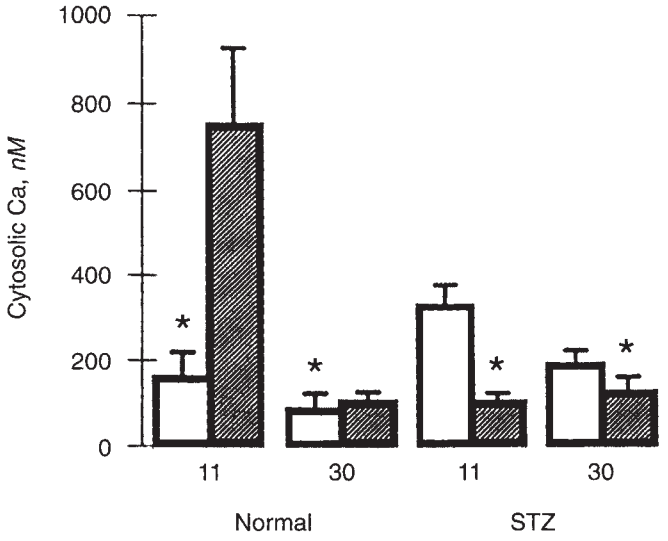

Fig. 5. *Effect of high glucose (30 mM Glc) on the response of cultured mesangial cells to Ang II.* Symbols are (□) minimal dose of Ang II (10^{-7} M); (▨) maximal dose of Ang II (10^{-5} M). All values are the means ± SE of cytosolic calcium concentration ($N = 6$) calculated from fluorescence measurements of suspended mesangial cells obtained from STZ-diabetic and normal animals cultured in the presence of physiological culture medium (11 mM) and high (30 mM) glucose (Glc); $^*P < 0.05$.

II-stimulated control cells (Fig. 3). The Figure shows that the cells were desensitized and had a transient pattern of elevation of cytosolic calcium, whereas no change in the type of response was observed with NOR in either control or diabetic groups (Figs. 6 and 7).

The initial increase of intracellular calcium depends on the activation of IP3 receptor in the sarcoplasmic reticulum, and is followed by calcium influx through the membrane channels stimulated by the influx of calcium released into the cytosol starting from the decrease in intracellular stores [12]. Putney [22] demonstrated that this influx of the sustained phase is independent of voltage-dependent channels and called it CRAC. Recently, Menè et al [20] described these channels in mesangial cells in culture and the channels were also observed in our laboratory during stimulation of mesangial cells with FK 506 [23]. The initial phase of calcium elevation remained unchanged in all experiments performed, demonstrating that the mesangial cells from both normal and diabetic animals have a similar preserved pathway of the increase in calcium concentration. Hurst, Whiteside and Thompson [24] demonstrated that the initial peak of IP3 formation was unchanged when the cells were exposed to high glucose concentrations, but that these levels were not maintained even after the activation of new receptors, suggesting a mechanism of down-regulation for IP3 synthesis. In our experiments, administration of a new Ang II dose did not promote an additional increase in cytosolic calcium, indicating that there were no reserve receptors available for activation, since it is known that the number of Ang II receptors in the glomeruli of diabetic animals is decreased, and that this response pattern reflects a phenotypic modification of the *in vitro* situation for Ang II receptors. Another possibility is that the number of receptors was unchanged but that down-regulation mechanisms were activated, such as an increase in the active PKC pool that might inhibit the synthesis of more IP3 by the activation of other membrane receptors. The site of action for this down-regulation is still doubtful but appears to be located in G protein, hindering separation of the α subunit from the $\beta\ \gamma$ subunit and thus allowing the activation of the effector which, under our conditions, was PLC [25, 26].

If it is reasonable to accept that desensitization results from PKC activation, one may wonder which is the activator factor since under our experimental conditions the desensitization phenomenon was present even in the absence of a medium with high glucose concentration, considering that these cells had been transferred to a culture medium with normal glucose concentrations after approximately three months. This suggests that there may be a change in the genetic load of the cell for the Ang II receptor acquired from the environment *in vivo*, from a non-treated diabetic medium in hyperglycemia, and expressed in culture regardless of the normal glucose concentrations of the medium. It is known that recovery of kidney lesions does not occur immediately after transplantation of islets [2] or of a diabetic kidney to a nondiabetic medium [27], clearly showing that some existing modifications last a longer time, perhaps due to a genetic memory independent of external conditions or to a slow turnover of glycosylated membrane proteins acquired for the chronic hyperglycemic medium.

This phenomenon of desensitization was specific for Ang II, supporting the hypothesis that the dysfunction may be located in the G protein linkage to the Ang II receptor and may not be due only to a decrease in the IP3 free pool by competition with glucose [7]. This may be a dysfunction shared by all agonist that utilize IP3

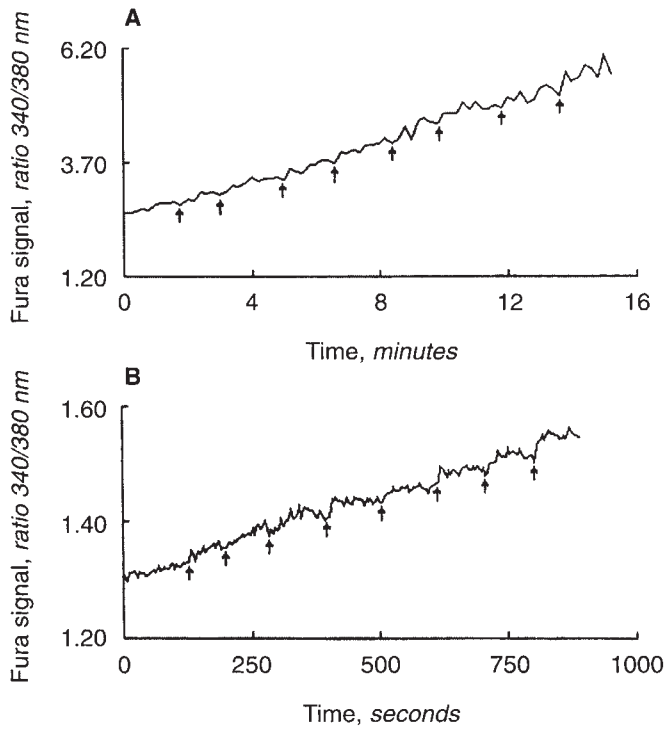

Fig. 6. *Comparison of the effects of norepinephrine (NOR) on cultured mesangial cells from normal Wistar rats grown in* (**A**) *physiological (11 mM) and* (**B**) *high (30 mM) glucose (Glc).* Both are representative traces of 6 experiments each of the cumulative dose response curves. Arrows indicate addition of NOR (10^{-9}, 5×10^{-9}, 10^{-8}, 5×10^{-8}, 10^{-7}, 5×10^{-7}, 10^{-6} and 5×10^{-6} M).

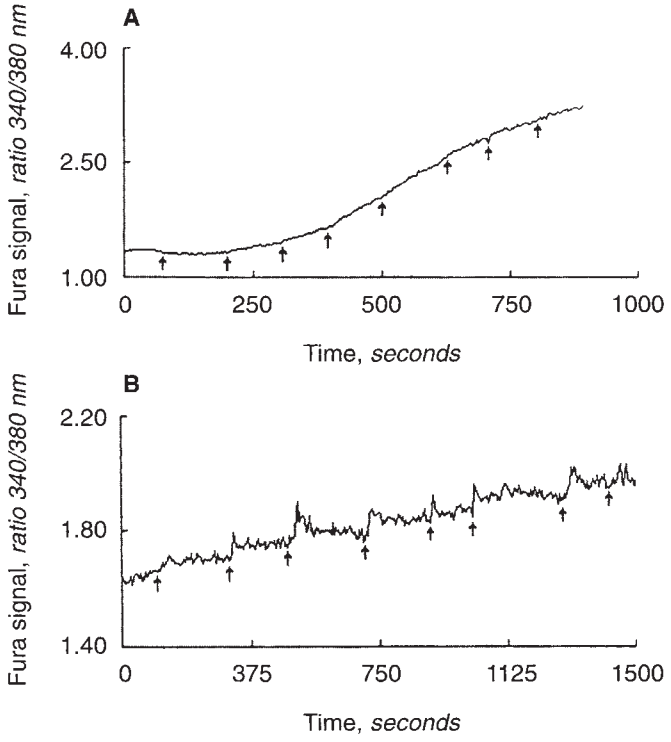

Fig. 7. *Comparison of the effects of norepinephrine (NOR) on cultured mesangial cells from STZ-diabetic rats grown in* (**A**) *physiological (11 mM) and* (**B**) *high (30 mM) glucose (Glc).* These traces are representative of 6 experiments each of cumulative dose response curves. Arrows indicate addition of NOR (10^{-9}, 5×10^{-9}, 10^{-8}, 5×10^{-8}, 10^{-7}, 5×10^{-7}, 10^{-6} and 5×10^{-6} M).

as a secondary messenger, since only a reduction in the response but not desensitization was observed with NOR.

The last interesting finding is that high glucose concentrations decreased the intensity of cytosolic calcium elevation in response to both Ang II and NOR (Figs. 5 and 8) and that this event was independent of the osmotic effect of glucose since mannitol did not promote these changes. It has been demonstrated that the use of PKC blockers reverses this situation [7], which was possibly also present in our experimental situation. This finding suggests that glucose may potentiate a pre-existing mechanism in diabetic cells even in an environment with normal glucose levels, since the response of diabetic cells to Ang II or NOR stimulation was lower than the response of control cells. Moreover, diabetic mesangial cells may have a lower functional capacity of elevating cytosolic calcium in response to both Ang II and NOR and that high glucose levels in the culture medium may enhance this dysfunction.

These results suggest that the MC from animals with induced diabetes acquired a phenotypic characteristic that was preserved *in vitro*, over several cell generations. It is also reasonable to propose a direct effect of STZ on MC since STZ was given in a single dose and after 30 to 180 days the animals were sacrificed and the glomeruli were cultured in order to obtain the MC. Since these MC were provided as a primary cultured cells (slow grown rate), they were studied after approximately five months (third subculture) under control culture conditions. Therefore, a long lag time of six to eight months occurred between STZ administration and the study period. Additionally, the toxic effects of STZ may elevate the intracellular calcium concentration in MC, but no significant difference in basal calcium was observed in cultured

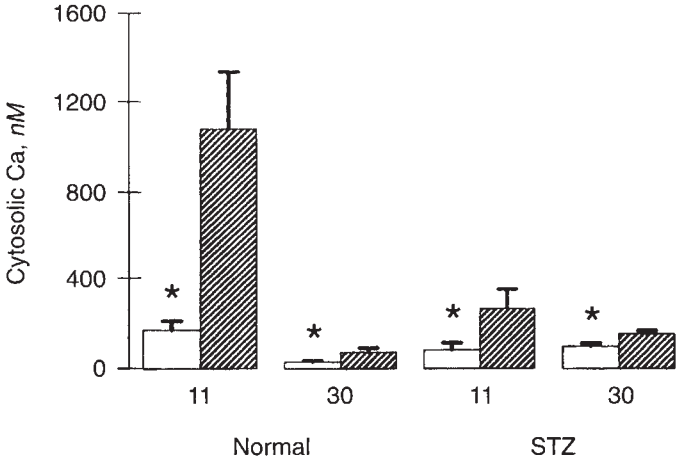

Fig. 8. *Effect of high glucose on the response of cultured mesangial cells to NOR.* Symbols are: (□) minimum dose of Nor (10^{-9} M); (▨) maximal dose of NOR (5×10^{-6} M). All values are the means ± SE of estimated cytosolic calcium concentration ($N = 6$) calculated from fluorescence measurements of suspended mesangial cells obtained from STZ-diabetic and normal animals, cultured in the presence of physiological culture medium (11 mM) and high (30 mM) glucose (Glc); $^*P < 0.05$.

MC from control and STZ rats. On the other hand, it is probable that the hyperglycemia and the insulin deficiency of STZ rats, among other *in vivo* milieu alterations, contribute to the striking differences observed in cultured MC from DM rats when compared with control MC. It is also important to emphasize that these MC were obtained from rats made diabetic and not treated with insulin, and were thus in a state of severe hyperglycemia with a strong impact on various hemodynamic and metabolic features. Therefore, these data indicate that the potential genetic changes induced by diabetes were transferred from *in vivo* to *in vitro* conditions, where they were maintained even after a long period of time (about 5 months) during which the cells were grown in normal culture medium.

This initial study opens new perspectives in the field of experimental investigation of the pathophysiology of diabetic nephropathy, suggesting that it is possible to study diabetic mesangial cells *in vitro* for a better understanding of their participation in the process of glomerular hyperfiltration and the hyperproduction of matrix components, which are among the principal characteristic and structural findings of diabetic nephropathy.

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